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Brief Report

Circulating endothelial progenitors are increased in Covid-19 patients and correlate with SARS-CoV-2 RNA in severe cases

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Essentials:

- Thrombotic phenomena and/or diffuse vascular damage are frequent in Covid-19, and viral elements have been observed within endothelial cells.
- Using a validated flow cytometry procedure, we found that viable circulating endothelial progenitors (CEPs) were significantly increased in Covid-19 patients compared to healthy controls.
- A positive correlation was found between the copies of SARS-CoV-2 RNA in the cellular fraction and apoptotic CEPs/mL in severe Covid-19 patients.
- CEPs might be investigated as candidate biomarkers of endothelial damage in Covid-19 patients.

Abstract

Background:

During the course of Covid-19, the disease caused by the new Coronavirus SARS-CoV-2, thrombotic phenomena and/or diffuse vascular damage are frequent, and viral elements have been observed within endothelial cells.

Objectives:

CD146+ circulating endothelial cells (CD146+ CECs) and their progenitors (CEPs) are increased in cardiovascular, thrombotic, infectious and cancer diseases. The present study was designed to investigate their kinetics in Covid-19 patients.

Methods:

We used a validated flow cytometry procedure to enumerate viable and apoptotic CD146+ CECs and CEPs in Covid-19 patients during the course of the disease and in patients who recovered.

Results:

Viable CEPs/mL were significantly increased in Covid-19 patients compared to healthy controls. This increase was observed in patients with mild symptoms and not further augmented in patients with severe symptoms. In patients who recovered, CEPs decreased, but were in a range still significantly higher than normal controls. Regarding mature CD146+ CECs, in Covid-19 patients their absolute number was similar to those observed in healthy controls, but the viable/apoptotic CD146+ CEC ratio was significantly different. Both mild and severe Covid-19 patients had significantly less apoptotic CD146+ CECs compared to healthy controls. Patients who recovered had significantly less CD146+ CECs/mL when compared to controls as well as to mild and severe Covid-19 patients. A positive correlation was found between the copies of SARS-CoV-2 RNA in the cellular fraction and apoptotic CEPs/mL in severe Covid-19 patients.

Conclusions:

CD146+ CECs and CEPs might be investigated as candidate biomarkers of endothelial damage in Covid-19 patients.

Key Words: Endothelial cells; Circulating Endothelial Cells; Circulating Endothelial Progenitors; Covid-19; SARS-CoV-2.

Introduction

The virus causing the 2020 coronavirus epidemic has been called "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2). The disease caused by the new Coronavirus has been called Covid-19 (1). During the course of the disease, and particularly in the advanced phase, thrombotic phenomena and/or diffuse vascular damage are frequently observed (2-4). Moreover, viral elements have been observed within endothelial cells in Covid-19 patients (5). An early assessment of these phenomena is believed to potentially help in selecting patients to be treated more quickly.

Circulating endothelial cells (CD146+ CECs) and their progenitor counterparts (CEPs) are endothelial cells present in the peripheral blood of healthy subjects (where apoptotic CD146+ CECs deriving from the turnover of the vascular endothelium are observed) and increased in cardiovascular, thrombotic, infectious and cancer diseases (6-7). A validated and reproducible procedure (8) allows to quantify CD146+ CECs and CEPs by multiparametric flow cytometry and to divide them into their viable and apoptotic fractions.

In the present work we measured CD146+ CECs and CEPs in active Covid-19 patients, subjects who had Covid-19, recovered and tested negative in the previous week, and age- and gender-matched healthy controls. SARS-CoV-2 RNA viremia was determined in the plasma and cellular fraction of every Covid-19 patient with digital droplet PCR, an assay known to reduce the frequency of false negatives when comparted to RT-PCR (9).

Patients and Methods

Patients and controls

During April 2020 we investigated 27 consecutive active Covid-19 patients (13 severe and 14 mild according to RCP values, see ref. 1), and 9 subjects who had Covid-19, recovered and tested negative in the previous week. There were 17 females and 19 males, median age was 52 (range 27-92). Eight age- and gender-matched healthy controls were also investigated.

Patient clinical and laboratory data were retrieved from the electronic clinical record. Laboratory data included whole blood cell count, RCP, D-Dimer, creatinine, IL-6, and ferritin.

Flow cytometry

CD146+ CECs and CEPs were evaluated by flow cytometry according to Mancuso et al (8) with little modifications. In brief, blood was collected in EDTA tubes as anticoagulant. After lysis of 7ml of blood with ammonium chloride (NH_4Cl), cell were incubated with monoclonal antibodies for 15 minute, at 4°C, in the dark . CD146+ CECs and CEPs were evaluated by ten-color flow cytometry (Navios EX, Beckman Coulter) using the nuclear staining Syto16 for DNA (Thermo Fisher Scientific, Eisai, Medipost – US), 7-AAD (BD) and a panel of monoclonal antibodies including anti-CD45 (to exclude hematopoietic

cells), anti CD34, anti-CD31, (both from Beckman Coulter) and anti-CD146 (BD) as endothelial cell markers. After acquisition of at least $3x10^6$ events, an appropriate gating strategy was used to enumerate viable and apoptotic CD146+ CECs and CEPs (Kaluza sotware, Beckman Coulter).

At the best of our knowledge, until now there is no a definitive consensus regarding mature CEC and CEP phenotype. According to some authors (10), CD146 is expressed on both CEPs and CECs. Some other authors (reviewed in 6-8, and 11) have restricted CEP clonogenic potential to DNA+, CD146-negative, CD45-negative, CD34+ cells.

In this work, CD146+ CECs were identified as DNA+, CD45-, CD31+, CD34+, CD146+ and CEPs as DNA+, CD45-, CD31+, CD34+, CD146-. Viable and apoptotic cells were identified by 7-AAD detection. The absolute number of the cells was calculated by a dual-platform counting technique using the total number of leukocytes in peripheral blood obtained by hemocytometer, according to routine methods.

Digital droplet PCR

RNA was extracted from 1200 ml of plasma and 600 ml of packed cellular fraction by QIAamp circulating nucleic acid kit (Qiagen,Germany) and tested by SARS-CoV-2 RNA digital droplet polymerase chain reaction (QX200, Bio-Rad) with CDC primers and probes (Integrated DNA technologies, 2019 nCOV-KIT, see ref. 9).

Ethical approval

The “Studio virologico, immunologico, clinico e genetico sui pazienti con infezione da nuovo coronavirus SARS-CoV-2” was approved with the registration number 2020/ST/049 at the “Sacco” Hospital in Milan.

Statistical analyses

Normal distribution of the data was assessed using Shapiro Wilk's normality test and the homogeneity of the variance was checked with the F-test for single comparison while using Bartelett's test for pairwise comparisons. Single and pairwise comparisons were performed using either the Student's t-test or the Wilcoxon's Rank Sum test according on normal or not normal distributions respectively; eventually the pairwise comparison p-values were adjusted by the Benjamini-Hochberg method.
For the correlations, Pearson or Spearman tests were employed based on normal or not normal distributions assessed again using Shapiro Wilk's normality test
Only significant differences ($p < 0.05$) were plotted. Statistical analyses and plots were carried out using ggpubr and ggplot packages on R. All the data and the scripts used for data analysis are available from Github (https://github.com/raveancic/endo_cells_COVID19)"

Results and Discussion

As shown in Fig. 1A-G, viable and apoptotic CEPs/mL were significantly increased in Covid-19 patients compared to healthy controls ($p<0.001$). This increase was larger than those usually observed in other neoplastic or vascular diseases (6-8). Interestingly, the increase was observed in patients with mild symptoms and not further augmented in patients with severe symptoms. Patients who recovered had less viable and apoptotic CEPs/mL, but in a range still significantly higher than normal controls ($p<0.05$).

Regarding mature CD146+ CECs, in Covid-19 patients their absolute number was similar to those observed in healthy controls, but the viable/apoptotic CD146+ CEC ratio was significantly different, as patients had less apoptotic CD146+ CECs ($p<0.001$). Again,

this difference was larger than those usually observed in other neoplastic or vascular diseases (6-8). Patients who recovered had significantly less apoptotic CD146+ CECs/mL when compared to controls ($p<0.001$) as well as to mild and severe Covid-19 patients ($p<0.01$).

SARS-CoV-2 RNA was found in the plasma and/or in the cellular fraction of 8 out of 13 severe Covid-19 patients (61%), and of 3 out of 14 mild Covid-19 patients (21%, $p=0.034$ by X^2 test). An interesting positive correlation ($R=0.80$, $p=0.001$) was found between the copies of SARS-CoV-2 RNA in the cellular fraction and apoptotic CEPs/mL in severe Covid-19 patients (Fig. 1H). Along a similar line, as shown in Fig. 2D, a positive correlation was found between viral RNA copies and the percentage of apoptotic CEPs ($R=0.6$, $p=0.02$). As also shown in Fig. 2, in severe Covid-19 patients other significant correlations were found between apoptotic CEPs and IL6, lymphocytes, RCP at entrance, total leukocyte count, lymphocytes, and monocytes. In this subgroup of patients, viable and/or apoptotic CD146+ CECs were found to correlate with RCP at enrollment and/or at the time of test.

As shown in Fig. 3, in patients affected by mild Covid-19, significant correlations were found between apoptotic CEPs and eosinophils, platelets, hematocrit, hemoglobin, IL6, and RCP at enrollment. Apoptotic CD146+ CECs correlated with viral RNA copies in plasma, hematocrit, hemoglobin, IL6, RCP at enrollment and the time of test.

As shown in Fig. 4, in patients who recovered after Covid-19, and tested negative in the week before, significant correlations were found between CD146+ CECs and red blood cell count, hemoglobin, MCV and MCH.

There is emerging evidence that cardiovascular complications are frequently a crucial step in Covid-19 progression and related deaths (1-4). The SARS-CoV-2 virus infects patients by mean of the ACE2 receptor, which is widely expressed in organs in the respiratory tract, as well as in the kidney, intestine, and in the cardiovascular system. Endothelial cells express high levels of the ACE2 receptor, and a recent report has shown evidence of direct viral infection of endothelial cells and diffuse endothelial inflammation in Covid-19 patients (5). To our knowledge, this is the first report showing that CEPs are significantly unbalanced in Covid-19 patients with a pattern that was not

previously reported in cancer, infectious and/or cardiovascular diseases. Nizzoli et al. (12) reported an increase in CD146+CECs in Covid-19 patients. However, in their work they did not present separate data about viable and apoptotic CD146+ CECs, and did not separate patients according to severity of their disease.

Data reported here suggest that already in patients affected by mild Covid-19 disease CD146+ CECs are significantly less apoptotic than in healthy controls, and that this unbalance is present also in severe Covid-19 patients. Both viable and apoptotic CEPs are significantly increased in mild and severe Covid-19 patients. Interestingly, Covid-19 patients who recovered from symptoms and tested negative in the week before had significantly less apoptotic CD146+ CECs when compared to controls and SARS-CoV-2-positive patients, whereas CEPs were still increased when compared to controls.

The mechanisms causing these unbalances deserve further translational investigation. Teuwen et al (13) and Atkermann et al (14) report that the lungs from patients with COVID-19 show distinctive vascular abnormalities which included severe endothelial injury associated with the presence of intracellular virus. Pulmonary vessels in such patients also showed widespread thrombosis and microangiopathy. Interestingly, in the lungs of patients with COVID-19 there was evidence of new blood vessel growth, i.e., angiogenesis, but not as a result of sprouting angiogenesis, but through a process known as ‘intussusceptive’ angiogenesis. The authors speculate that this burst in intussusceptive angiogenesis may be the result of an adaptive response to the hypoxia associated with ARDS in such COVID patients.

The correlation found between copies of SARS-CoV-2 RNA and apoptotic CEPs, if confirmed by cell sorting, suggest the hypothesis that also these progenitors, in addition to mature endothelial cells, might be a direct target of this pathogen. According to Monteil et al (15), SARS-COV-2 virus can directly infect blood vessels in 3-dimentional kidney organoids in cell culture. A damage inflicted on the pulmonary endothelium would result in an increase in apoptotic CECs, whereas the adaptive angiogenesis response may do the opposite and result in an increase in CEPs. SARS-CoV-2-related endothelial damage might have triggered CEP mobilization from the bone marrow (or other

reservoirs such as the white adipose tissue, see ref. 16-17) in an attempt to generate a newly functional vessel lining. These hypotheses should be investigated in adequate preclinical models.

There is ongoing interest in undertaking clinical trials of antiangiogenic drugs such as anti-VEGF antibodies as a possible treatment strategy for COVID-19 patients. The rationale is that VEGF can act as a potent permeability factor that causes vessel leakage (12-13), and hence may be responsible for the pulmonary edema associated with ARDS in COVID-19 patients. Should in the future be possible to pinpoint circulating endothelial cells originating from the lung or different organs, this piece of information will be of great interest to better understand their role in Covid-19.

Taken together, our findings indicate that CD146+ CECs and CEPs deserve further study as possible candidate biomarkers of endothelial damage in Covid-19 patients, particularly in the early phases of the disease. This hypothesis is currently under investigation in our Institutions, and might pave the way to clinical trials using these biomarkers for patient selection and/or stratification.

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Figure Legends

Fig. 1: Features of CEP and CD146+ CEC in Covid-19 patients.

A-G) Distributions of CEP and CD146+ CEC values in the patient groups (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001).

H) Significative correlation of apoptotic CEP and viral RNA copies in the cellular fraction.

Fig. 2: Significant correlations among CEP and CD146+ CEC categories and hematological parameters in severe Covid-19 patients.

Fig.3: Significant correlations among CEP and CD146+ CEC categories and hematological parameters for the in mild Covid-19 patients.

Fig. 4: Significant correlations among CEP and CD146+ CEC categories and hematological parameters in recovered Covid-19 patients.

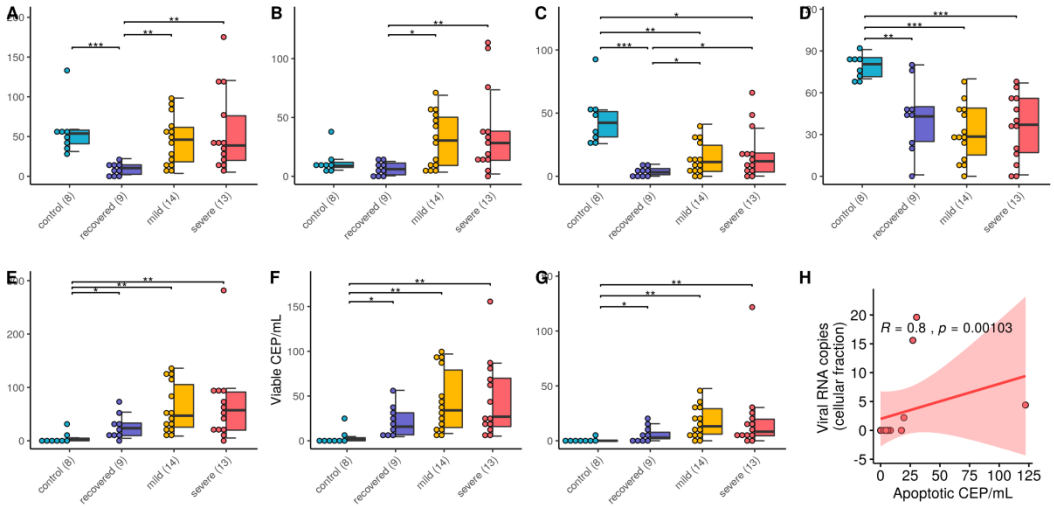
Author Contribution:

PM, AG, and FB designed the study

PM, AG, GG, PC, JQ, SC, LG, SR, and AG performed the study

AR analyzed the data

PM, AG, CC, and FB wrote the manuscript



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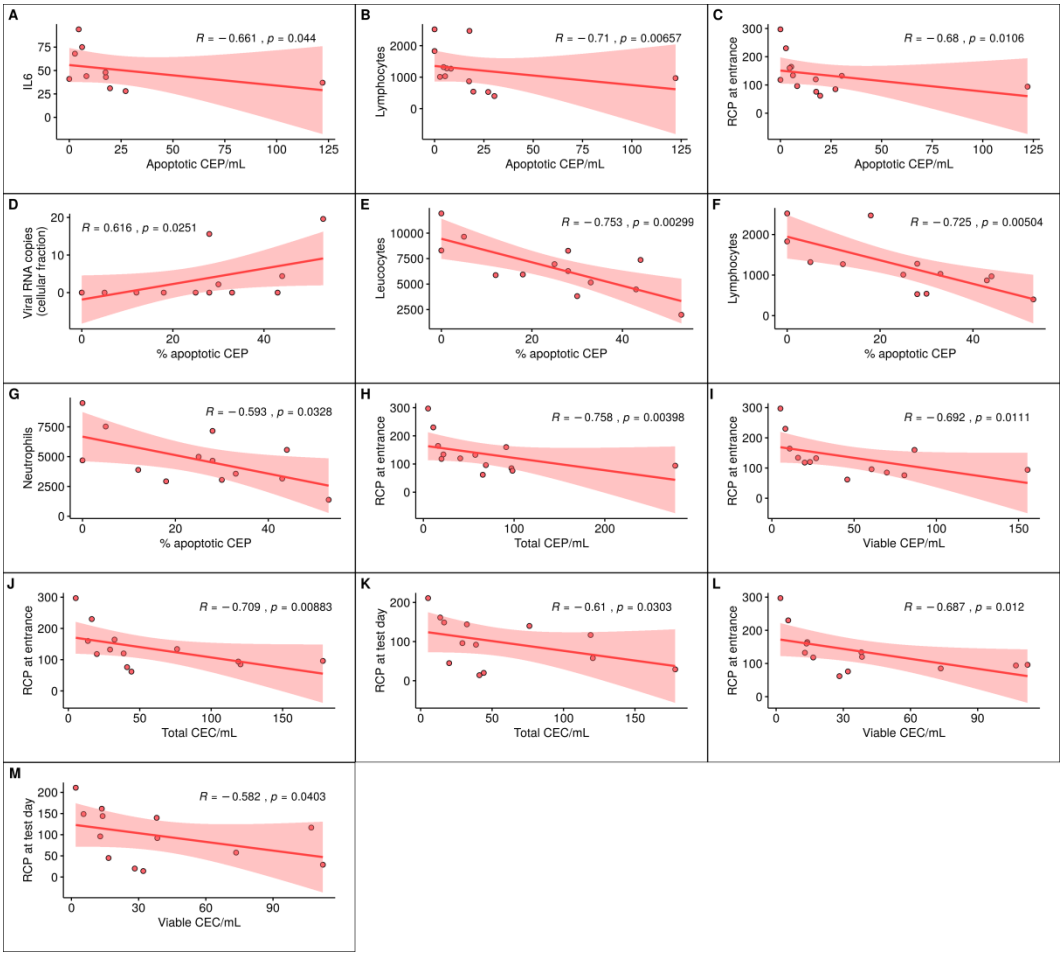

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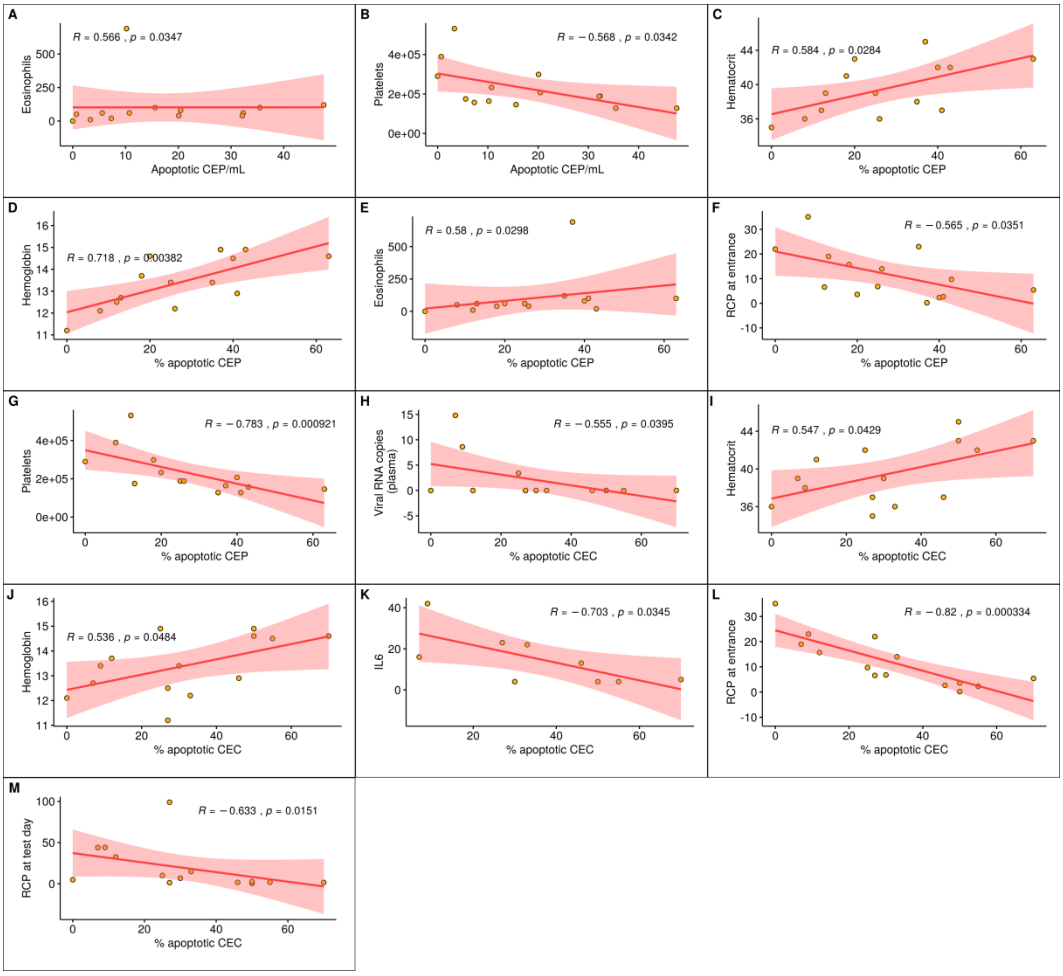

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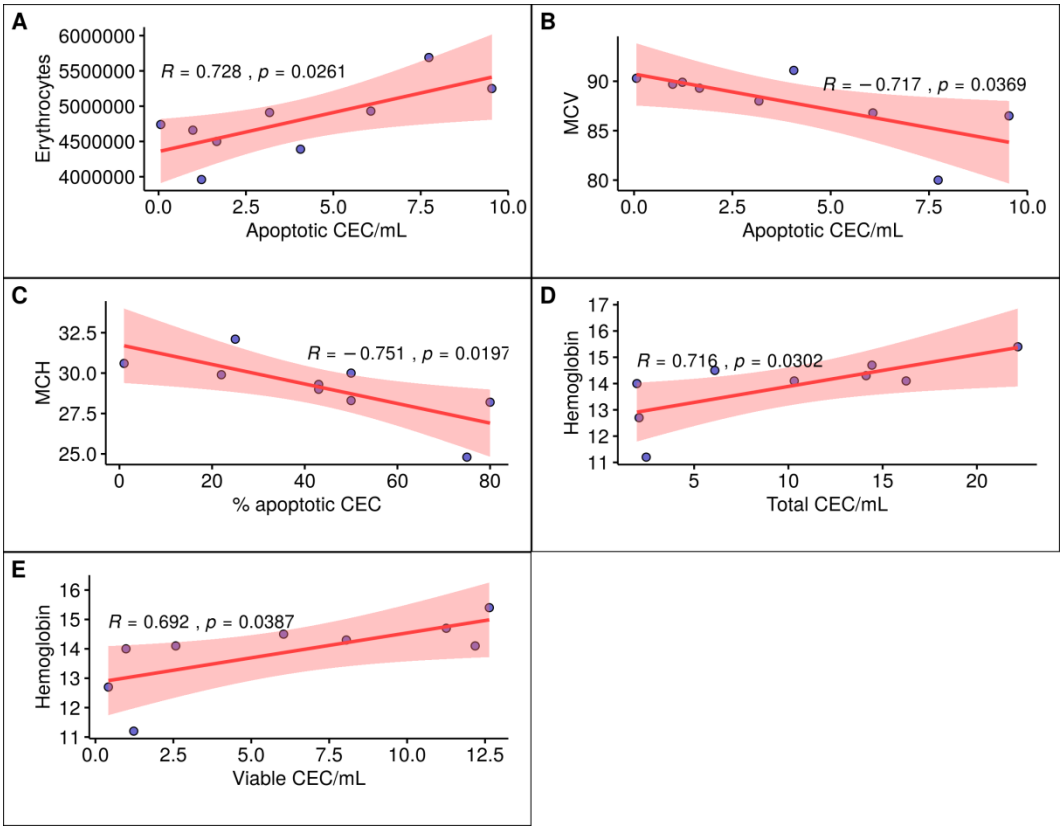

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